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# STUDIES ON BACTERIAL NUTRITION

## THE UTILIZATION OF NITROGENOUS COMPOUNDS OF DEFINITE CHEMICAL COMPOSITION

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### INTRODUCTION AND REVIEW OF LITERATURE

The recent studies on bacterial nutrition have emphasized the important part played by the simple nitrogenous compounds in furnishing a readily available source of nitrogen. Some knowledge of the rôle played by these substances has been attained as a result of a number of very important and interesting contributions to this subject.

The work of Bainbridge (1911) demonstrated that purified unaltered animal proteins, when supplied as the sole source of nitrogen, failed to furnish the necessary conditions for the development of certain aerobic and facultative anaerobic bacteria. This was substantiated by Sperry and Rettger (1915) who showed, in addition, that the putrefactive anaerobes were unable to attack native proteins and that pure vegetable proteins, as edestin, exhibited the same resistance to direct bacterial attack. They considered this resistance to be due to the complex construction of the protein molecule, which rendered it unfit for immediate utilization.

Rettger, Berman, and Sturges (1916) found that coagulated albumin shows the same resistance to the direct action of bacteria as do the unchanged native proteins. They also demonstrated that proteose and peptones are not attacked, or at least very slowly, by the gelatin nonliquefying species. On the other hand, the organisms elaborating a proteolytic enzyme accomplished the destruction of these substances quite readily.

Attention will be directed in this paper to the amino-acids and other simple nitrogenous compounds, for it is to these substances that one must turn to find sources of immediately available nitrogen.

Several investigators have obtained striking results by employing protein-free mediums composed of a mixture of the products of enzymatic protein cleavage. Dalimier and Lancereaux (1913) made use of a product known commercially as opsine. They succeeded in cultivating not only the commoner saprophytes but also many of the more exacting pathogens, as B. typhosus, B. diphtheriae, B. anthracis, B. tetani, B. tuberculosis (human, bovine and avian), the pneumococcus, meningococcus, and gonococcus.

Robinson and Rettger (1918), in a more extensive study of opsine, were able to cultivate the obligate anaerobes, the Welch bacillus and many other exacting pathogens, in addition to the above-mentioned organisms. They also found that better results were obtained with the enzymatic digestion products of pro-

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tein, opsine, than with the acid hydrolysis products of edestin, lactalbumin, or casein.

However, when one or more chemically definite nitrogenous compounds have been substituted for the above heterogeneous mixtures, the results have not been so promising. Uschinsky's medium (Uschinsky 1893 a, b) and Fränkel's modification (1894) support a good growth with many of the commoner saprophytes and a few of the less exacting pathogens, but both fall far short of constituting an ideal definite chemical culture medium.

Numerous studies have been made of the ability of various species of bacteria to utilize chemically-definite nitrogenous foodstuffs when supplied as the only source of nitrogen. Thus, the tubercle bacillus has been studied by Proskauer and Beck (1894), Frouin (1912), Armand-Delille et al. (1913), Tiffeneau and Marie (1912), and by Magoon (1916), all of whom have reported successful cultivation on various mediums of a known chemical structure. Bielecki (1911) investigated the development of the anthrax bacillus in solutions of amino-acids. Hadley (1907) claimed to have succeeded in obtaining growth and toxin production by B. diphtheriae in a "synthetic" medium. Dolt (1908) prepared several simple "synthetic" mediums for growing the colon bacillus and recommends two of these as suitable for water analysis. Clark and Lubs (1917) recently recommended a medium consisting of anhydrous $Na_2HPO_4$ 0.7%; KH-phthalate, 0.2%; aspartic acid, 0.1%, and dextrose, 0.4%, for the differentiation of the colon-aerogenes group. Both B. coli and B. aerogenes grew well and no diminution in the growth or vigor of fermentation was observed on a number of successive transfers through the above medium.

Zunz and György (1916) studied the influence of proteoses, peptids, amino-acids, purins, urea, and extractive bodies on the development and indol production by B. coli and B. typhosus. Sasaki (1912 a) found that B. coli was able to effect a splitting of the dipeptids glycyl-tyrosin and glycyl-glycin. Later (1912 b, c) he extended these observations and obtained the same results with various members of the colon-typhoid-dysentery group, Micrococcus tetragenus and 12 species of gelatin liquefiers. In another publication (1914) he demonstrated the production of p-oxyphenylethylamin from tyrosin by B. coli in a chemically-definite medium.

Jordan (1899) employed a number of "synthetic" mediums for the purpose of studying the production of fluorescent pigment by bacteria. Blanchetière (1917) investigated the mode and conditions governing the attack on asparagin by Bacillus fluorescens liquefaciens. Franzen and Egger (1914) employed definite chemiccal mediums to determine the relative nutritional value of glycocoll, alanin, and asparagin for B. prodigiosus. Nawiasky (1908) studied the ability of Proteus vulgaris to attack various amino-acids, asparagin, creatin, taurin, uric acid, and urea. Frouin and Ledebt (1912) cultivated B. pyocyaneus on a medium in which asparagin constituted the only source of nitrogen.

The use of "synthetic" mediums for the purpose of isolating from the intestine certain bacteria which attack the products of digestion has been suggested by several workers. Among these should be mentioned Berthelot (1911, 1912, and 1917) who has recommended several suitable mediums and has studied a number of the isolated organisms. Mellanby and Twort (1912) isolated a bacillus of the colon group which possessed the power of converting histidin, by decarboxylation, into ergamin. Jones (1918) records the isolation of an organism called Bacillus aminophilus which was capable of producing this same product from histidin. A similar organism had been previously described by Berthelot and Bertrand (1912).

In the foregoing publications attention has been centered, for the most part, on a single species of bacterium. In contrast to the many investigations of such nature which occur in the literature, one finds that comparatively few attempts have been made to determine the relative values of the various simple and chemically-definite nitrogenous foodstuffs for different species. This field of investigation would seem to be an important one, for by obtaining a knowledge of the availability of these substances we might hope to construct, eventually, a medium of known chemical structure on which even the more exacting pathogens would develop. The advantages of such a medium for the study of metabolism, toxin production, the structure of toxins, etc., are obvious. At the present time, however, we are far from realizing this ideal.

As a step in this direction Lepièrre (1903) investigated the availability of certain glucoproteins. The glucoproteins were added in quantities of 1.5-3 gm. to a medium composed of:

| | |
|---|---|
| Water | 100 c.c. |
| NaCl | 0.5 gm. |
| $MgSO_4$ | 0.05 gm. |
| Calcium glycerophosphate | 0.2-0.3 gm. |
| $KHCO_3$ | 0.1-0.2 gm. |

Only the simpler glucoproteins containing 6-11 carbon atoms were employed. In some cases glucose, saccharose, or glycerol, was added as an additional source of carbon. Of 45 different micro-organisms tested by Lepièrre, 14 pathogenic and 23 saprophytic species were able to develop on all the glucoproteins, while B. diphtheriae, B. anthracis, B. tetani, a species of streptococcus, the vibrion septique and B. tuberculosis required glucoproteins containing 8-11 carbon atoms. In the light of more recent knowledge, however, it would seem that Lepièrre's glucoproteins were not as definite structurally as he supposed.

Sullivan (1906), employed many different "synthetic" mediums in his study on bacterial pigments, but the number of nitrogenous compounds used was somewhat limited. After testing the ability of various pigment producers to develop on his mediums he recommends one composed of asparagin, 1%; magnesium sulphate, 0.02%; di-potassium hydrogen phosphate, 0.1%; ammonium lactate, 0.05%; sodium chlorid, 0.5%; potassium nitrate, 0.02%; glycerol, 1%, and agar, 2%.

Probably the most noteworthy study of the availability of nitrogenous compounds of definite chemical structure is that of Galimard and Lacomme (1907). These authors employed as a substratum the inorganic salts used by Lepièrre plus 1.5% of glycerol. The nitrogen was supplied by various amino-acids and urea, added either singly or in combinations. In this way glycocoll, leucin, aspartic acid, tyrosin, phenylalanin, lysin, arginin, and urea were subjected to the action of 24 different species of bacteria. Arginin was found to be superior as a single source of nitrogen, since 13 of the 24 species were able to develop when this amino-acid was present. Phenylalanin failed entirely to support growth, while lysin and aspartic acid each supported the growth of but a single species—B. pyocyaneus. Urea supported the growth of but 2 species while glycocoll, leucin, and tyrosin gave somewhat better results.

In addition, 11 different combinations of the above amino-acids were tried. One medium composed of glycocoll, 0.3%; leucin, 0.05%; tyrosin, 0.01%; aspartic acid, 0.1%, and arginin chlorhydrate, 0.23%, and a second of glycocoll, 0.9%, and arginin chlorhydrate, 0.1% proved to be the 2 most satisfactory combinations. It is noteworthy that B. diphtheriae, B. tetani, B. tuberculosis, B. typhosus, and several species of cocci failed to develop in every case.

Quite recently Gordon (1917) attempted to ascertain whether the commoner pathogenic bacteria exhibit any marked difference in regard to their individual capacity to break down certain nitrogenous substances of known chemical composition.

He employed a medium consisting of:

| | |
|---|---|
| $K_2HPO_4$ | 0.1% |
| $MgSO_4$ | 0.2 |
| $CaCl_2$ | 0.01 |
| NaCl | 0.5% |
| Washed agar | 3% |
| Various ammonium salts, amides, or amino-acids (unless only sparingly soluble) | 1% |

This medium was tried both with and without the addition of glucose. It was found that where glucose was present as a source of carbon, bacteria, such as B. coli, B. paratyphosus, B. pyocyaneus, and B. proteus can satisfy their nitrogen requirement with either ammonium salts, sundry amides, amino-acids (glycocoll, alanin, aspartic acid) or asparagin. On the other hand, B. typhosus, Friedländer's bacillus, B. diphtheriae, B. pseudo-diphtheriae, and several species of staphylococci and streptococci appear to possess no such ability. The cholera vibrio and B. dysenteriae (Flexner) were found to be capable of utilizing certain of the amino-acids, but not the ammonium salts.

Summing up, we see that attention is being more and more centered on the simple nitrogenous bodies instead of those of complex nature, and that promising results have been secured by employing a heterogeneous mixture of the products of proteolytic cleavage. Hence, the present need would appear to be a thorough study of the isolated and purified products of protein hydrolysis.

The aim of the present investigation has been to gain some knowledge of the ability of various species of bacteria to utilize these simpler nitrogenous substances of known chemical structure. Chief emphasis has been placed on the amino-acids, the purins and extractives, and various combinations of these substances.

## EXPERIMENTAL

The following combination was adopted as the basic medium:

| | |
|---|---|
| Distilled ammonia-free water | 1,000 c.c. |
| NaCl | 5.0 g. |
| $MgSO_4$ | 0.2 g. |
| $CaCl_2$ | 0.1 g. |
| $KH_2PO_4$ | 1.0 g. |
| $K_2HPO_4$ | 1.0 g. |
| Glycerol | 30.0 g. |

To this constant substratum the nitrogenous bodies to be tested were added, usually in quantities sufficient to make 0.1%.

This constant substratum is essentially a modification of Uschinsky's medium from which the nitrogen-containing compounds—aspargin and ammonium lactate—have been withdrawn and the amount of glycerol reduced to 3%. The primary potassium phosphate was added to neutralize the alkaline reaction imparted by the di-potassium phosphate. Several determniations of the hydrogen ion concentration, made by the colorimetric method, showed that the medium described in the foregoing had a $P_H$ value of 6.6.

Glycerol was used as a source of energy, as its presence was found to favor the growth of the various organisms. Dextrose—1%—was also tried and, with a few minor exceptions, gave essentially the same results as were secured with glycerol. On the whole, there appeared to be somewhat less acid formed from the glycerol, and as this allowed greater freedom of development it was given preference over the dextrose.

To this constant substratum were added the various simple nitrogenous compounds, either singly or in combinations. The resultant medium is clear and colorless and does not require filtration.* It was filled into ordinary test tubes and sterilized in the autoclave at 10 or 12 lbs. extra pressure for 15 minutes. Liquid mediums only were employed.

Only those nitrogenous compounds which were of undoubted purity were used. The writers are indebted to Prof. F. P. Underhill for the following: valin, glutamic acid, phenylalanin, tyrosin, lysin picrate, urea, uric acid, hypoxanthin, allantoin, and creatin. Glycocoll, asparatic acid, and asparagin, were procured from the Eimer and Amend Co. The tryptophan, obtained from the same company, was labeled Grübler and Co., Leipzig. To prepare leucin, the writers hydrolyzed serum globulin (horse) with sulphuric acid. The leucin fraction was separated and the leucin purified after the method of Ehrlich and Wendel (1908). Histidin monochlorid was prepared from sheep blood according to Knoop (1907). Lysin dichlorid was obtained from lysin picrate after the method of Kossel (1898). Taurin was prepared from desiccated ox-bile (Hammarsten and Hedin, 1914). In every case the products thus secured were subjected to repeated crystallization to insure their purity.

All inoculations, unless otherwise indicated, were made with the tip of a bent platinum rod from 24-hour slant agar cultures of the micro-organisms in question, care being taken to introduce as small a quantity as possible of the culture.

Several experiments in which inoculations were made from old agar cultures showed that the results obtained were variable and unreliable, due presumably to the introduction of enzymes, dead bacterial cells, or other nitrogenous matter into the synthetic medium.

Precautions were taken to prevent absorption of atmospheric ammonia during the period of incubation. As free ammonia may be given off from cultures in the ordinary peptone meat mediums during the course of bacterial development, all tubes of the various "synthetic" mediums, when placed in an incubator containing other cultures, were enclosed in small metal boxes to prevent, in so far as possible, the absorption of gasseous ammonia.

The McFarland nephelometer was employed in estimating luxuriance of growth. Standards were made to give the following densities: 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, and 7.0. It was found unnecessary to go above the last figure. These terms represent the actual number of c c of the barium chlorid solution added to the sulphuric acid. Five c c amounts of the standard suspensions were filled into test tubes of a diameter equal to that of the tubes used to contain the culture mediums. All standard tubes were then sealed in a flame to prevent evaporation.

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* The turbidity which is quite apparent while the medium is hot completely disappears on cooling.

To estimate the amount of growth the culture in question was compared with those tubes of the standard series most nearly approximating it. The comparison was made against a black background in strong indirect light. Although this method, as used in the present investigation, does not give an accurate estimate of the number of bacterial cells in a given culture, it nevertheless gives a series of gradients which express fairly accurately the degree of turbidity resulting from the growth of the micro-organisms in question. It possesses the advantage of furnishing definite chemical standards which do not deteriorate and by means of which the results obtained with varying nitrogenous compounds and by different investigators may be more readily correlated. In the case of a flocculent, stringy, or "mucilaginous" growth, comparison is made with difficulty or cannot be made at all. This was found to be the case with cultures of B. cereus, B. subtilis, and B. mycoides and also to a certain extent with the older cultures of B. pyocyaneus, B. cloacae, B. acidi (lactici) and occasionally others. Nevertheless, in the writers' opinion, this method has proved to be on the whole quite reliable and much to be preferred to the expression of growth by the terms, slight, good, etc.

To gain an idea of the rapidity of development, readings were taken by comparison with the standards at intervals of 24 hours, 48 hours, 4 days and 7 days, after inoculation. In a number of cases the period of observation was extended to 2 weeks, but as little additional information was gained by this procedure it was discontinued.

To check the first series of results in any given medium, transplants were made from these tubes into a second series of the same medium. This inoculation was made within the first 24 hours—wherever sufficient growth had been attained to permit this—by means of a small platinum loop about 1 mm. in diameter. To further check the results stains were made and agar slants stroked from the cultures showing no growth or in those cases where the results were questionable.

## MONO-AMINO ACIDS AS THE ONLY SOURCE OF NITROGEN

The following mono-amino acids were employed: glycocoll, valin, leucin, aspartic acid, and glutamic acid. All were used in 0.1% concentration. In addition, the glycocoll and aspartic acid were made up into 0.3% solutions and submitted to the same culture tests. The aspartic and glutamic acid mediums were rendered neutral to litmus by the addition of sodium hydroxid solution. Since the results obtained with the different mono-amino acids are essentially the same, only 1 of the 7 complete tables is presented here (see Table 1).

B. subtilis, B. cereus and B. mycoides produced flocculent growths, so it was impossible to determine their luxuriance by the barium sulphate standards. The extent of growth is expressed by + or ++ signs, the former indicating slight and the other fair growth.

Several of the organisms employed are conspicuous because of their inability to develop on any of the mediums containing the above amino-acids. They are B. anthracis, Proteus zenkeri, B. typhosus, B. dysenteriae, B. abortus, B. pullorum, B. diphtheriae, B. hoffmanni and all of the cocci studied. Of the pathogens employed only B. pyocyaneus, and paratyphoid bacilli, A and B, B. sanguinarium, and the cholera vibrio were able to utilize the amino-acids as a source of nitrogen. The common saprophytic bacilli exhibited for the most

Note.—For additional tables and other data not included in this paper the reader is referred to the doctorate thesis (S. A. Koser) in the Yale University Library.

part an abundant growth, although the spore-formers, B. subtilis, B. cereus and B. mycoides developed rather slowly, scantily and irregularly.

The inability of leucin to function as an important source of nitrogen for bacteria is of particular interest, in view of the recent publication of Zunz and György (1916). As a result of their own investigation these authors claim that leucin promotes luxuriant development of B. coli, and that B. typhosus finds sufficiently favorable conditions for slight but unmistaken growth. In our experience leucin has been in no wise different from glycocoll or any of the other mono-amino acids employed.

TABLE 1

CONSTANT SUBSTRATUM PLUS 0.1% GLYCOCOLL

| | Degree of Growth at Expiration of | | | |
|---|---|---|---|---|
| | 24 Hrs. | 48 Hrs. | 4 Days | 7 Days |
| B. cereus (Frankland) | 0.0 | +? | + | + |
| B. subtilis (Cohn) | 0.0 | ? | + | + |
| B. mycoides (Flügge) | 0.0 | ? | + | + |
| B. anthracis | 0.0 | 0.0 | 0.0 | 0.0 |
| B. fluorescens liq. | 0.25 | 1.5 | 3.0 | 4.0 |
| B. fluorescens nonliq. | 0.5 | 1.0 | 1.5 | 2.0 |
| B. pyocyaneus | 0.5 | 1.0 | 2.0 | 2.0 |
| B. prodigiosus | 0.5 | 1.0 | 1.5 | 2.0 |
| B. ruber balticus | 0.0 | 0.25 | 1.0 | 1.5 |
| Proteus vulgaris | 0.0 | 0.25 | 0.25 | 0.25* |
| Proteus mirabilis | ? | 0.25 | 0.25 | 0.25 |
| Proteus zenkeri | 0.0 | 0.0 | 0.0 | 0.0 |
| B. capsulatus | 0.5 | 1.0 | 1.0 | 1.0 |
| B. coli (104) | 0.75 | 1.0 | 1.5 | 1.5 |
| B. coli (U) | 0.5 | 1.0 | 1.5 | 2.0 |
| B. aerogenes | 0.5 | ..... | 4.0 | 5.0 |
| B. cloacae | 0.5 | 1.0 | 1.5 | 3.0 |
| B. acidi lactici | 0.5 | 1.0 | 1.5 | 2.0 |
| B. paratyphosus A | 0.5 | 0.75 | 0.75 | 1.0 |
| B. paratyphosus B | 0.25 | 0.75 | 1.0 | 1.5 |
| B. pullorum | 0.0 | 0.0 | 0.0 | 0.0 |
| B. sanguinarium | 0.0 | 0.0 | 0.25 | 0.25 |
| B. typhosus | 0.0 | 0.0 | 0.0 | 0.0 |
| B. dysenteriae (Shiga) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. abortus (Bang) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. abortus (Wis.) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. hoffmanni | 0.0 | 0.0 | 0.0 | 0.0 |
| B. diphtheriae (Park No. 8) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. diphtheria (Y. M. S.) | 0.0 | 0.0 | 0.0 | 0.0 |
| V. cholerae (R) | 0.0 | ? | 1.0 | 2.0 |
| Sarcina lutea | 0.0 | 0.0 | 0.0 | 0.0 |
| Sarcina aurantiaca | 0.0 | 0.0 | 0.0 | 0.0 |
| Mic. tetragenus | 0.0 | 0.0 | 0.0 | 0.0 |
| Mic. cereus flavus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. aureus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. albus | 0.0 | 0.0 | 0.0 | 0.0 |
| Str. pyogenes (309) | 0.0 | 0.0 | 0.0 | 0.0 |
| Str. pyogenes (346) | 0.0 | 0.0 | 0.0 | 0.0 |
| Streptothrix | 0.0 | 0.0 | + | + |

* Failed to grow on reinoculation into 2nd tube.

Uschinsky's medium was employed for the purpose of comparison. The results with all of the test organisms were so nearly like those furnished by the above experimental mediums that they are not presented here in tabulated form.

A full set of control tests was made also with the constant substratum. All of the organisms failed to develop except in a very few instances in which on prolonged incubation a very light or questionable growth occurred, in all probability as the result of the absorption of minute quantities of ammonia from the atmosphere. The few organisms which seemed to possess this ability were later found to appropriate to their own needs the ammonia nitrogen of ammonium phosphate.

In a medium containing free aspartic or glutamic acid as the only source of nitrogen the results are very uncertain, as is shown also in the conflicting data which have been reported from other laboratories recently. When such a medium is neutralized the aspartic or glutamic acid will play essentially the same rôle as glycocoll, leucin, etc.

TABLE 2

SHOWING THE EFFECT OF DECREASING THE SUPPLY OF NITROGEN

Aspartic Acid (neutralized) Added to the Usual Constant Substratum in Varying Amounts. Record of Growth Made at Expiration of 7 Days

| | None (Control) | 0.1% | 0.02% | 0.002% | 0.001% | 0.0001% |
|---|---|---|---|---|---|---|
| B. cereus | ..... | ++ | + | + | ? | 0.0 |
| B. subtilis | ..... | + | + | ? | 0.0 | 0.0 |
| B. mycoides | ..... | + | + | ? | ? | 0.0 ? |
| B. fluorescens | 0.0 | 1.5 | 1.5 | 0.75 | 0.25 | 0.0 |
| B. fluorescens nonliq. | ..... | 3.0 | 2.0 | 0.5 | 0.25 | ? |
| B. pyocyaneus | 0.0 ? | 1.5 | 1.5 | 0.75 | 0.25 | ? |
| B. prodigiosus | ..... | 4.0 | 1.5 | 0.5 | ? | 0.0 |
| Proteus mirabilis | ..... | 0.5 | 0.25 | 0.25 | 0.0 | 0.0 |
| B. capsulatus | 0.0 | 5.0 | 1.5 | 0.75 | ? | 0.0 |
| B. coli (104) | 0.0 | 3.0 | 1.5 | 0.5 | 0.0 | 0.0 |
| B. coli (U) | ..... | 4.0 | 1.5 | 0.5 | ? | 0.0 |
| B. cloacae | 0.0 | 4.0 | 1.5 | 0.5 | 0.25 | 0.0 ? |
| B. acidi | 0.0 | 5.0 | 1.5 | 0.5 | ? | 0.0 |
| B. paratyphosus A | ..... | 1.5 | 1.5 | 0.5 | ? | 0.0 |
| B. paratyphosus B | ..... | 1.5 | 1.5 | 0.5 | ? | 0.0 |
| B. sanguinarium | ..... | 1.0 | 0.5 | 0.25 | ? | 0.0 |
| V. cholerae (R) | ..... | 1.5 | 0.5 | 0.5 | 0.0 | 0.0 |
| V. cholerae (Y. M. S.) | 0.0 | 1.0 | 1.0 | 0.5 | 0.0 | 0.0 |

A concentration of 0.1% of the mono-amino acids supplies sufficient nitrogen for the organisms which can utilize it at all, since the results were practically the same whether the amino-acids constituted 0.1 or 0.3% of the medium. In order to obtain further information as to the minimum amount of amino-acid required to visibly affect the luxuriance of bacterial growth, aspartic acid was employed in diminishing quantities, namely, 0.1%, 0.02%, 0.002%, 0.001%, 0.0001% (Table 2). As the amount of amino-acid nitrogen was decreased bacterial multiplication was lessened until at a concentration of 0.0001% there was little if any development. Certain organisms which in 0.1% aspartic acid attain a luxuriance of growth of 3.0, 4.0 or 5.0, are unable to develop beyond 1.5 on the barium sulphate scale when the aspartic acid is decreased to 0.02%. All of these tests were made with neutralized aspartic acid medium, using, as at all other times, the substratum described earlier in this paper.

## VIABILITY EXPERIMENTS IN AMINO-ACID MEDIUMS

The following plan was adopted for determining the ability of bacteria to preserve their normal growth characteristics during long periods of cultivation in the amino-acid mediums. Ten representative organisms were selected and carried through 12 successive cultures in 0.1% aspartic acid medium rendered neutral to litmus. The inoculations from tube to tube were made with a 1 mm. loop, and at inetrvals of 24 hours, or, in the case of the slowly-growing forms, as soon thereafter as sufficient growth was obtained.

In Table 3 are given figures for the first, eighth, and the twelfth or last of the consecutive cultures. As no diminution in the luxuriance of growth had occurred up to this time further cultures were not made. The last set of cultures was kept at room temperature for a period of 3 months. Slants of nutrient agar were streaked from these tubes every 2 weeks, with the following results.

TABLE 3

SHOWING THE RESULT OF CONSECUTIVE TRANSPLANTS IN AMINO-ACID MEDIUM:

0.1% Aspartic Acid—Neutralized

| | 24 Hrs. | 48 Hrs. | 4 Days | 7 Days |
|---|---|---|---|---|
| 1st Culture: | | | | |
| B. cereus | 0.0 | +? | ++ | ++ |
| B. fluorescens | 0.5 | 1.5 | 3.0 | 4.0 |
| B. pyocyaneus | 0.5 | 1.0 | 1.5 | 1.5 |
| B. prodigiosus | 0.25 | 1.5 | 2.0 | 3.0 |
| Proteus vulgaris | 0.0 | 0.0 | 0.0 ? | 0.0 ? * |
| B. coli (U) | 0.5 | 1.5 | 3.0 | 4.0 |
| B. paratyphosus B | 0.5 | 1.0 | 1.0 | 1.0 |
| B. cloacae | 0.5 | 2.0 | 3.0 | 3.0 |
| B. acidi | 1.0 | 2.0 | 3.0 | 4.0 |
| V. cholerae (R) | 0.0 | ? | 0.5 | 1.0 |
| 8th Consecutive Culture: | | | | |
| B. cereus | ..... | ? | + | + |
| B. fluorescens | 1.0 | 2.0 | ..... | 4.0 |
| B. pyocyaneus | 0.5 | 1.5 | 1.5 | 1.5 |
| B. prodigiosus | 0.5 | 2.0 | 2.0 | 3.0 |
| Proteus vulgaris | 0.0 | 0.0 | 0.0 | 0.0 * |
| B. coli (U) | 2.0 | 3.0 | 4.0 | 5.0 |
| B. paratyphosus B | 0.5 | 1.0 | 1.0 | 1.0 |
| B. cloacae | 1.0 | 2.0 | 3.0 | 3.0 |
| B. acidi | 1.0 | 1.5 | 4.0 | 5.0 |
| V. cholerae (R) | ..... | 0.25 | 1.0 | 1.0 |
| 12th Consecutive Culture: | | | | |
| B. cereus | ..... | + | ++ | ++ |
| B. fluorescens | 0.5 | 1.5 | 3.0 | 5.0 |
| B. pyocyaneus | 1.0 | 2.0 | 2.0 | 2.0 |
| B. prodigiosus | 1.0 | 1.5 | 2.0 | 2.0 |
| Proteus vulgaris | 0.0 | 0.0 | 0.0 | 0.0 * |
| B. coli (U) | 1.5 | 4.0 | 4.0 | 5.0 |
| B. paratyphosus B | 0.5 | 1.0 | 1.5 | 1.0 |
| B. cloacae | 1.0 | 2.0 | 3.0 | 3.0 |
| B. acidi | 1.5 | 3.0 | 4.0 | 5.0 |
| V. cholerae (R) | ..... | 0.5 | 1.5 | 1.5 |

* Good growth was obtained when agar slants were stroked from the cultures at 7th day.

B. paratyphosus B and the cholera vibrio were found to have died by the end of the 2nd week, while B. coli and B. acidi lactici failed to grow when transplanted to the agar after a month. These 4 organisms produced sufficient acid, however, from the glycerin in the constant substrate to give a positive reaction with methyl red. The remaining amino-acid cultures, which gave only a pure yellow or neutral tint with methyl red, remained viable to the termination of the experiment, as did control cultures of the same organisms in bouillon.

Repeated transplants in the amino-acid medium did not result in any apparent loss of viability. Indeed, in a few instances, growth became more luxuriant after each successive transfer.

## THE RELATIVE SIGNIFICANCE OF LARGE AND SMALL AMOUNTS OF INOCULUM ON RAPIDITY AND LUXURIANCE OF GROWTH

In the ordinary transfer of bacteria from one medium to another by means of the platinum straight wire or loop, hundreds of thousands, and at times millions of living bacterial cells are carried over, in spite of all possible care. After some preliminary experiments it was found that by inoculating tubes of sterile water and making certain dilutions, it was possible to transfer, with the aid of a 4 mm. loop, fairly definite numbers, 200-600 cells.

TABLE 4

CONSTANT SUBSTRATUM PLUS 0.1% LYSIN

Lysin Dichlorid 0.15%—Neutralized with $Na_2CO_3$

| | Degree of Growth at Expiration of | | | |
|---|---|---|---|---|
| | 24 Hrs. | 48 Hrs. | 4 Days | 7 Days |
| B. cereus | 0.0 | + | + | + |
| B. subtilis | 0.0 | 0.0 | 0.0 | 0.0 |
| B. ramosus | + | + | + | + |
| B. anthracis | 0.0 | 0.0 | 0.0 | 0.0 |
| | | | | |
| B. fluorescens | 0.5 | 1.0 | 2.0 | 3.0 |
| B. fluorescens nonliq. | 0.5 | 1.0 | 3.0 | 4.0 |
| B. pyocyaneus | 1.0 | 1.0 | 1.5 | 2.0 |
| B. prodigiosus | ? | 0.5 | 1.0 | 1.5 |
| B. ruber | 0.0 | 0.0 | 0.5 | 1.5 |
| | | | | |
| Proteus vulgaris | 0.0 | ? | 0.25 | 0.25 |
| Proteus mirabilis | 0.25 | 0.5 | 0.5 | 0.5 * |
| Proteus zenkeri | 0.0 | 0.0 | 0.0 | 0.0 |
| | | | | |
| B. capsulatus | 0.5 | 1.0 | 1.5 | 2.0 |
| B. coli (104) | 0.5 | 0.75 | 1.0 | 1.5 |
| B. coli (U) | 0.5 | 1.0 | 1.0 | 2.0 |
| B. aerogenes | 0.25 | 0.5 | 1.0 | 2.0 |
| B. cloacae | 1.0 | 1.0 | 1.5 | 2.0 |
| B. acidi lactici | 1.0 | 1.0 | 1.0 | 1.5 |
| B. paratyphosus A | 0.5 | 0.5 | 1.0 | 1.0 |
| B. paratyphosus B | ? | 0.5 | 0.5 | 1.0 |
| B. pullorum | 0.0 | 0.0 | 0.0 | 0.0 |
| B. sanguinarium | 0.0 | 0.0 | 0.0 | 0.0 |
| B. typhosus | 0.0 | 0.0 | 0.0 | 0.0 |
| B. dysenteriae (Shiga) | 0.0 | 0.0 | 0.25 | ? |
| | | | | |
| B. abortus (Bang) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. abortus (Wis.) | 0.0 | 0.0 | 0.0 | 0.0 |
| | | | | |
| B. hoffmanni | 0.0 | 0.0 | 0.0 | 0.0 |
| B. diphtheriae (Park No. 8) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. diphtheriae (Y. M. S.) | 0.0 | 0.0 | 0.0 | 0.0 |
| | | | | |
| V. cholerae (R) | 0.5 | 1.0 | 1.0 | 1.0 |
| V. cholerae (Y. M. S.) | 0.0 | 0.0 | 0.25 | 1.0 |
| | | | | |
| Sarcina lutea | 0.0 | 0.0 | 0.0 | 0.0 |
| Sarcina aurantiaca | 0.0 | 0.0 | 0.0 | 0.0 |
| Mic. tetragenus | 0.0 | 0.0 | 0.0 | 0.0 |
| Mic. flavus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. aureus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. albus | 0.0 | 0.0 | 0.0 | 0.0 |
| Str. pyogenes 309 | 0.0 | 0.0 | 0.0 | 0.0 |
| Str. pyogenes 346 | 0.0 | 0.0 | 0.0 | 0.0 |
| | | | | |
| Streptothrix | 0.0 | 0.0 | + | ++ |

* Failed to develop when reinoculated into 2nd tube.

Two different amino-acid mediums, the one containing valin, and the other aspartic acid as the sole source of nitrogen, were each inoculated, by the above dilution method, with the different test organisms. As large inoculums are accompanied by appreciatble amounts of enzymes, in case they are elaborated, as has been shown in this laboratory, the smallest amounts that were practicable were employed. These experiments were repeated several times.

TABLE 5

CONSTANT SUBSTRATUM PLUS 0.1% PHENYLALANIN

| | Degree of Growth at Expiration of | | | |
|---|---|---|---|---|
| | 24 Hrs. | 48 Hrs. | 4 Days | 7 Days |
| B. cereus | ? | + | ++ | ++ |
| B. subtilis | 0.0 | 0.0 | + | + |
| B. ramosus | 0.0 | 0.0 | ++ | ++ |
| B. anthracis | 0.0 | 0.0 | 0.0 | 0.0 |
| B. fluorescens | 0.5 | 1.0 | 1.0 | 1.5 |
| B. fluorescens nonliq. | 0.25 | 0.5 | 0.5 | 1.0 |
| B. pyocyaneus | 0.25 | 0.5 | 1.0 | 1.0 |
| B. prodigiosus | 0.0 | ? | 0.5 | 1.0 |
| B. ruber | 0.0 | ? | 0.5 | 1.5 |
| Proteus vulgaris | 0.0 | 0.0 | 0.0 | ? |
| Proteus mirabilis | 0.0 | ? | 0.25 | 0.25* |
| Proteus zenkeri | 0.0 | 0.0 | 0.0 | 0.0 |
| B. capsulatus | 0.5 | 1.0 | 1.0 | 1.0 |
| B. coli (104) | 0.25 | 0.5 | 0.5 | 1.0 |
| B. coli (U) | ? | 0.25 | 0.5 | 0.5 |
| B. cloacae | 0.25 | 0.5 | 1.0 | 1.0 |
| B. acidi (lactici) | 0.25 | 0.25 | 1.0 | 1.0 |
| B. paratyphosus A | 0.25 | 0.5 | 0.5 | 0.5 |
| B. paratyphosus B | ? | ? | 0.5 | 0.5 |
| B. pullorum | 0.0 | 0.0 | 0.0 | 0.0 |
| B. sanguinarium | 0.0 | ? | ? | ? |
| B. typhosus | 0.0 | 0.0 | 0.0 | 0.0 |
| B. dysenteriae (Shiga) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. abortus (Bang) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. abortus (Wis.) | | | | |
| B. hoffmanni | 0.0 | 0.0 | 0.0 | 0.0 |
| B. diphtheria (Park No. 8) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. diphtheria (Y. M. S.) | | | | |
| V. cholerae (R) | 0.0 | 0.0 | 0.0 | 0.5 |
| V. cholerae (Y. M. S.) | | | | |
| Sarcina lutea | 0.0 | 0.0 | 0.0 | 0.0 |
| Sarcina aurantiaca | 0.0 | 0.0 | 0.0 | 0.0 |
| Mic. tetragenus | 0.0 | 0.0 | 0.0 | 0.0 |
| Mic. cereus flavus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. aureus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. albus | 0.0 | 0.0 | 0.0 | 0.0 |
| Str. pyogenes 346 | 0.0 | 0.0 | 0.0 | 0.0 |
| Streptothrix | 0.0 | 0.0 | ? | ? |

* On subculture to 2nd tube, growth was so slight as to be questionable.

By comparison with the results shown in Table 1 and similar tables not included in this paper, it was readily seen that during the first 24-48 hours the growth resulting from the small inoculum is not as luxuriant as that following the usual method. The relatively few cells which are carried over are able, however, to develop, and at the end of several days produce as luxuriant growth as is obtained with the larger inoculums, with the exception of Proteus vulgaris, which failed to grow, and of the subtilis group, which multiplied very slowly or not at all. The other organisms seem to be able to assimilate the amino-acid nitrogen without the aid of enzyme action, particularly B. capsulatus, B. coli, B. cloacae and B. acidi-lactici.

TABLE 6

CONSTANT SUBSTRATUM PLUS 0.1% HISTIDIN
Used as Histidin Monochlorid 0.135% and Neutralized

| | Luxuriance of Growth at Expiration of | | | |
|---|---|---|---|---|
| | 24 Hrs. | 48 Hrs. | 4 Days | 7 Days |
| B. cereus | 0.0 | + | ++ | ++ |
| B. subtilis | 0.0 | 0.0 | ..... | ? |
| B. ramosus | 0.0 | 0.0 | ..... | + |
| B. anthracis | 0.0 | 0.0 | 0.0 | 0.0 |
| B. fluorescens | 0.5 | 1.5 | 3.0 | 4.0 |
| B. fluorescens nonliq. | 0.5 | 1.0 | 3.0 | 5.0 |
| B. pyocyaneus | 1.0 | 1.5 | 1.5 | 2.0 |
| B. prodigiosus | 0.5 | 1.5 | 2.0 | 2.0 |
| B. ruber | 0.0 | 0.25 | 1.5 | 2.0 |
| Proteus vulgaris | 0.0 | ? | ? | 0.25*† |
| Proteus mirabilis | 0.0 | 0.0 | 0.0 | 0.0 * |
| Proteus zenkeri | 0.0 | 0.0 | 0.0 | 0.0 |
| B. capsulatus | 1.0 | 1.5 | 1.5 | 2.0 |
| B. coli (104) | 0.25 | 0.25 | 0.5 | 0.5 |
| B. coli (U) | 0.25 | 0.25 | 0.5 | 0.5 † |
| B. aerogenes | 0.75 | 2.0 | ..... | 2.0 |
| B. cloacae | 0.5 | 1.5 | 2.0 | 3.0 |
| B. acidi | 0.0 | ? | 0.25 | 0.5 |
| B. paratyphosus A | 0.5 | 0.75 | 1.0 | 1.5 |
| B. paratyphosus B | 0.5 | 0.5 | 1.0 | 1.5 |
| B. pullorum | 0.0 | 0.0 | 0.0 | 0.0 |
| B. sanuinarium | 0.25 | 0.25 | 0.25 | 0.25 |
| B. typhosus | 0.0 | 0.0 | 0.0 | 0.0 |
| B. dysenteriae (Shiga) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. abortus (Bang) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. abortus (Wis.) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. hoffmanni | 0.0 | 0.0 | 0.0 | 0.0 |
| B. diphtheriae (Park No. 8) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. diphtheriae (Y. M. S.) | 0.0 | 0.0 | 0.0 | 0.0 |
| V. cholerae (R) | 0.25 | 0.5 | 1.0 | 1.5 |
| V. cholerae (Y. M. S.) | 0.0 | 0.0 | 0.0 | 0.25 |
| Sarcina lutea | 0.0 | 0.0 | 0.0 | 0.0 ? |
| Sarcina aurantiaca | 0.0 | 0.0 | 0.0 | 0.0 |
| Mic. tetragenus | 0.0 | 0.0 | 0.0 | 0.0 |
| Mic. flavus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. aureus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. albus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. citreus | 0.0 | 0.0 | 0.0 | 0.0 |
| Strep. pyogenes (U) | 0.0 | 0.0 | 0.0 | 0.0 |
| Cladothrix | 0.0 | ? | ..... | + |

* Yellow.
† Failed to develop when reinoculated into 2nd tube.

## DIAMINO, AROMATIC, AND HETEROCYCLIC AMINO-ACIDS AS SOURCES OF NITROGEN

The substances comprising this mixed group were lysin, tyrosin, phenylalanin, histidin, and tryptophan.

Since lysin possesses 2 amino groupings in the molecule, a study of its availability as a source of nitrogen appeared promising. In Table 4 are given the results obtained with a medium containing lysin and the substrate employed throughout the investigation. Contrary to expectations, lysin apparently has no advantage over the mono-amino acids, at least in so far as its deportment toward the more exacting pathogenic species of bacteria is concerned. B. dysenteriae grew very slightly in one instance, but in subsequent tests the results were negative.

The availability of tyrosin and phenylalanin is represented in Table 5. The response to the different organisms employed was somewhat less than that secured with the mono-amino-acid mediums (See Table 1). A marked difference is noticeable in the luxuriance of the growth. For instance, with both strains of the colon bacillus and the paratyphoid organisms a less dense growth is obtained by the use of these aromatic amino-acids than with the mono-amino. The same is true of the chromogenic forms. It should be stated here that the quantity of tyrosin in solution in the tyrosin medium (0.03%) was limited by its slight solubility, and may in a measure at least be responsible for the lighter growth in this medium. Both tyrosin and phenylalanin are distinctly inferior to the other amino-acids employed in this work.

The heterocyclic amino-acid, histidin, is of no greater value as a source of available nitrogen than the mono-amino acids and lysin (Table 6). A peculiar coloration of this medium was brought about by Proteus vulgaris and P. mirabilis. During several days' incubation the medium assumed a light color which by the end of a week became a rich yellow. A similar phenomenon was observed when the histidin was replaced by tryptophan. Cornish and Williams (1917) had called attention to light pinkish brown or amber color production by the proteus group in a histidin or tryptophan medium.

Tryptophan appears to possess a slight advantage over the nitrogenous substances thus far considered. In addition to the organisms which usually develop in the amino-acid mediums, others produced a visible clouding. In some instances, however, ability to grow was lost on further transplantation in the same medium. This was particularly true of B. anthracis, B. dysenteriae, and Sarcina aurantiaca. On the other hand, B. typhosus was capable of producing light growth through the several successive transfers.

Proteus vulgaris and P. mirabilis brought about a distinct yellow to amber color in the tryptophan medium after several days' incubation. A very light color was apparent also in both cultures of the cholera vibrio in the same medium. This observation indicates a similarity in the nature of the decomposition of tryptophan and histidin, at least in so far as the unusual color product is concerned.

## OTHER SOURCES OF NITROGEN

In addition to the various substances employed in the preceding experiments a number of other chemically-definite sources of nitrogen were submitted to the same tests, namely, ammonium phosphate, urea, taurin, creatin, hypoxanthin, uric acid and allantoin.

Diammonium hydrogen phosphate yielded results but slightly different from those obtained with the amino-acids. Even such organisms as the paratyphoid bacilli and B. sanguinarium are able to satisfy their nitrogen requirements in the presence of this ammonium salt. However, both strains of the cholera vibrio refused to grow, although the experiment was repeated several times. This observation is in accordance with that of Gordon (1917) who states that the cholera vibrio is capable of utilizing certain amino-acids, but not ammonium salts.

Urea is somewhat inferior to the amino-acids as nitrogenous food for bacteria, while taurin and creatin failed to supply available nitrogen in almost every case.

TABLE 7

URIC ACID, 0.1%, ADDED TO CONSTANT SUBSTRATUM, FROM WHICH THE 0.1% $KH_2PO_4$ WAS OMITTED

| | Degree of Growth at Expiration of | | | |
|---|---|---|---|---|
| | 24 Hrs. | 48 Hrs. | 4 Days | 7 Days |
| B. cereus | 0.0 | 0.0 | 0.0 | 0.0 |
| B. subtilis | 0.0 | 0.0 | 0.0 | 0.0 |
| B. ramosus | 0.0 | 0.0 | 0.0 | 0.0 |
| B. anthracis | 0.0 | 0.0 | 0.0 | 0.0 |
| B. fluorescens | 0.5 | 1.5 | 3.0 | 4.0 |
| B. fluorescens nonliq. | 0.25 | 0.5 | 2.0 | 3.0 |
| B. pyocyaneus | 0.25 | 1.0 | 1.5 | 1.5 |
| B. prodigiosus | 0.5 | 1.0 | 1.5 | 1.5 |
| B. ruber | 0.0 | 0.0 | 0.5 | 1.0 |
| Proteus vulgaris | 0.0 | 0.0 | 0.0 | 0.0 |
| Proteus mirabilis | 0.0 | 0.0 | 0.0 | 0.0 |
| Proteus zenkeri | 0.0 | 0.0 | 0.0 | 0.0 |
| B. capsulatus | 0.5 | 1.0 | 2.0 | 2.0 |
| B. coli (104) | 0.0 | 0.0 | 0.0 | 0.0 ? |
| B. coli (U) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. aerogenes | 0.25 | 1.0 | 3.0 | 5.0 |
| B. cloacae | 0.5 | 1.5 | 2.0 | 3.0 |
| B. acidi | 0.0 | 0.0 | 0.0 | 0.0 |
| B. paratyphosus A | 0.0 | ? | ? | ? |
| B. paratyphosus B | 0.0 | 0.0 ? | 0.0 ? | ? |
| B. pullorum | 0.0 | 0.0 | 0.0 | 0.0 |
| B. sanguinarium | 0.0 | 0.0 | 0.0 | 0.0 |
| B. typhosus | 0.0 | 0.0 | 0.0 | 0.0 |
| B. dysenteriae (Shiga) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. abortus (Bang) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. abortus (Wis.) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. hoffmanni | 0.0 | 0.0 | 0.0 | 0.0 |
| B. diphtheriae (Park No. 8) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. diphtheriae (Y. M. S.) | 0.0 | 0.0 | 0.0 | 0.0 |
| V. cholerae (R) | 0.0 | 0.0 | 0.0 | 0.0 |
| V. cholerae (Y. M. S.) | 0.0 | 0.0 | 0.0 | 0.0 |
| Sarcina lutea | 0.0 | 0.0 | 0.0 | 0.0 |
| Sarcina aurantiaca | 0.0 | 0.0 | 0.0 | 0.0 |
| Mic. tetragenus | 0.0 | 0.0 | 0.0 | 0.0 |
| Mic. flavus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. aureus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. albus | 0.0 | 0.0 | 0.0 | 0.0 |
| Str. pyogenes (309) | 0.0 | 0.0 | 0.0 | 0.0 |
| Str. pyogenes (346) | 0.0 | 0.0 | 0.0 | 0.0 |
| Streptothrix | 0.0 | ? | ? | + |
| Cladothrix | 0.0 | + | + | ++ |

In order to determine whether the presence of taurin and creatin exert an inhibitive influence glycocoll was added to these mediums. The results were essentialy the same as when glycocoll alone was added to the substratum. Taurin and creatin are, therefore, merely indifferent, or resistant to bacterial action in the concentrations employed (0.1%).

The purins hypoxanthin and uric acid were next employed, as was also allantoin, an oxidation product of uric acid. The results obtained with hypoxanthin and uric acid were comparable, as might be anticipated, owing to their similarity in structure. Of the two agents the uric acid assumes the more importance, because certain differences which are brought about in both of these mediums are most accentuated by the uric acid. Table 7 presents the results of the uric acid experiments. It will be seen that certain organisms which grew in the amino-acid mediums failed to attack the uric acid, or at the most underwent but slight development in its presence. These are the subtilis and the proteus groups, B. coli, B. acidi lactici, the paratyphoid bacilli, B. sanguinarium and the cholera vibrio. The uric acid and hypoxanthin are inferior, therefore, to the amino-acids as sources of available nitrogen.

The points of special significance in the uric acid table is the sharp differentiation between the reactions of B. coli and B. acidi lactici on the one hand, and B. aerogenes and B. cloacae on the other. The former leave the medium unclouded, whereas B. aerogenes and B. cloacae produce luxuriant growths. In view of the recent interest attached to these types of organisms a more extended investigation was conducted on this method of their differentiation. The results have been published elsewhere.[1] Further experimentation has confirmed the earlier results, and the value of the uric acid synthetic medium in the separation of the coli and the aerogenes types of organisms appears to us to have been fully established. The results obtained with the uric acid medium are definitely correlated with the methyl red and the Voges-Proskauer reactions.

As uric acid is insoluble in water, but soluble in alkalies, it was necessary to omit the primary potassium phosphate from the constant substratum. By this procedure a 0.1% solution of uric acid, or rather potassium urate, could be obtained.

Although structurally quite similar to uric acid, allantoin yields entirely different results, deporting itself in about the same manner as the amino-acids. Presumably the free amino grouping in the allantoin renders this substance a more acceptable source of nitrogen than the purins in which all of the nitrogen is held in the purin ring.

## COMBINATIONS OF TWO OR MORE OF THE FOREGOING NITROGENOUS SUBSTANCES

The chemically-definite mediums employed up to this point contained only one nitrogen-containing ingredient, with the single exception of the Uschinsky solution. In the following experiments the culture medium was enriched by one or more additional nitrogenous substances. It was assumed that such combinations would tend to satisfy the nitrogen requirements of bacteria better than when the choice of nitrogen is more limited.

The first combination was one of glycocoll, aspartic acid and tyrosin. The same organisms were employed in this and subsequent experiments as heretofore. Contrary to expectations, the results were in nowise different from those obtained with the single nitrogenous substances, as for example glycocoll.

A combination of aspartic acid and lysin, or of aspartic acid, lysin and histidin, likewise proved to be of no added advantage.

In the next test creatin was added to a medium which already contained glycocoll, lysin and histidin. A careful comparison of the results with those obtained with the single nitrogenous substances showed them to be practically identical.

Finally, a mixture of the following agents was used: glycocoll, 0.05%; valin, 0.05%; aspartic acid, 0.05%; glutamic acid, 0.05%; tyrosin, 0.03%; a trace of lysin dichlorid; histidin monochlorid, 0.07%; tryptophan, 0.05%;

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[1] Koser, S. A.: Jour. Infect. Dis., 1918, 23, p. 377.

taurin, 0.05%; creatin, 0.05%, and allantoin, 0.05%. The resultant solution (in the constant substratum described in the early part of this paper) was rendered neutral to litmus by the addition of normal sodium hydroxid. The results are presented in Table 8.

TABLE 8

CONSTANT SUBSTRATUM PLUS MIXTURE OF 11 DIFFERENT NITROGENOUS SUBSTANCES (SEE PAGE ?)

| | Degree of Growth at Expiration of | | | |
|---|---|---|---|---|
| | 24 Hrs. | 48 Hrs. | 4 Days | 7 Days |
| B. cereus | + | ++ | +++ | +++ |
| B. subtilis | + | ++ | ++ | ++ |
| B. ramosus | + | ++ | ++ | ++ |
| B. anthracis | 0.0 | 0.0 | 0.0 | 0.0 |
| B. fluorescens | 1.0 | 2.0 | 2.0 | 2.0 |
| B. fluorescens nonliq. | 0.75 | 3.0 | 4.0 | 4.0 |
| B. pyocyaneus | 1.5 | 1.5 | 1.0 | 1.0 |
| B. prodigiosus | 2.0 | 2.0 | 3.0 | 4.0 |
| B. ruber | 1.0 | 2.0 | 3.0 | 4.0 |
| Proteus vulgaris | 0.25 | 0.25 | 0.25 | 0.25* |
| Proteus mirabilis | 0.25 | 0.5 | 0.5 | 0.5 * |
| Proteus zenkeri | ? | ? | 0.25 | 0.25† |
| B. capsulatus | 1.5 | 1.5 | 3.0 | 5.0 |
| B. coli (104) | 1.5 | 4.0 | 5.0 | 5.0 |
| B. coli (U) | 0.0 | 0.0 | 2.0 | 3.0 |
| B. aerogenes | 2.0 | 4.0 | 5.0 | 7.0 |
| B. cloacae | 1.5 | 4.0 | 3.0 | 5.0 |
| B. acidi lactici | 2.0 | 3.0 | 4.0 | 5.0 |
| B. paratyphosus A | 0.5 | 0.75 | 0.75 | 0.75 |
| B. paratyphosus B | 0.5 | 0.5 | 0.75 | 1.0 |
| B. pullorum | 0.25 | 0.25 | 0.25 | 0.25 |
| B. sanguinarium | 0.5 | 1.0 | 1.0 | 1.0 |
| B. typhosus | 0.5 | 0.5 | 0.5 | 0.75 |
| B. dysenteriae (Shiga) | 0.0 | 0.0 | 0.0 ? | 0.0 ? |
| B. abortus (Bang) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. abortus (Wis.) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. hoffmanni | 0.0 | 0.0 | 0.0 | 0.0 |
| B. diphtheriae (Park No. 8) | 0.0 | 0.0 | 0.0 | 0.0 |
| B. diphtheriae (Y. M. S.) | 0.0 | 0.0 | 0.0 | 0.0 |
| V. cholerae (R) | 0.5 | 0.5 | ..... | 0.75 |
| V. cholerae (Y. M. S.) | 0.25 | 1.0 | ..... | 1.5 |
| Sarcina lutea | 0.0 | + | ++ | ++ |
| Sarcina aurantiaca | 0.0 | 0.0 | 0.0 | 0.0 |
| Mic. tetragenus | 0.0 | 0.0 | 0.0 | 0.0 |
| Mic. flavus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. aureus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. albus | 0.0 | 0.0 | 0.0 | 0.0 |
| Staph. citreus | 0.0 | 0.0 | 0.0 | 0.0 |
| Str. pyogenes (U) | 0.0 | 0.0 | 0.0 | 0.0 |
| Streptothrix | 0.0 | + | ++ | ++ |
| Cladothrix | 0.25 | 0.5 | ++ | ++ |

* Deep yellow.
† Failed to develop when reinoculated into 2nd tube.

The development in this very complex medium of those organisms which grow in the simple nitrogenous mediums is somewhat accelerated, especially within the first 24-48 hours. Furthermore, B. typhosus, B. pullorum and Sarcina lutea were able to multipy in this medium. These organisms were carried through several successive cultures with no apparent diminution of growth. Several strains of B. typhosus were used, all giving the same results.

Proteus zenkeri exhibited a very slight growth in the first culture, but was unable to develop when transferred to a second tube. In every instance where histidin was present Proteus vulgaris and Proteus mirabilis produced the peculiar yellow color that has previously been mentioned. It is to be noted that in this complex medium, as in the simpler solutions, B. anthracis, B. abortus, B. diphtheriae, B. Hoffmanni and all but one of the coccoid forms are unable to develop.

## DISCUSSION

A general survey of the present investigation leads to the conclusion that the various amino-acids are quite similar in their ability to support the growth of certain micro-organisms. We see that certain species appear to be able to utilize and to synthesize their own protoplasm as readily from a very simple amino-acid, glycocoll, as from histidin or tryptophan. On the other hand, a number of organisms, as B. abortus and B. diphtheriae, are evidently unable to assimilate directly any of the amino-acids, extractives, or other sources of nitrogen which were offered.

The aerobic spore-formers, B. cereus, B. subtilis and B. mycoides, develop rather scantily and slowly in the amino-acid mediums. At times the growth is irregular and may fail to appear. The anthrax bacillus refused to grow in every case. The two species of fluorescent bacilli, B. pyocyaneus, B. prodigiosus and B. ruber balticus, develop readily when any one of the amino-acids, diammonium acid phosphate, hypoxanthin, uric acid, or allantoin, is supplied as the only source of nitrogen. The colon bacillus and closely allied organisms are also able to utilize the amino-acids, ammonium acid phosphate, urea and allantoin. B. capsulatus, B. cloacae and B. aerogenes apparently can attack hypoxanthin and uric acid, whereas the colon bacillus lacks this power. The paratyphoid bacilli appear to be quite "cosmopolitan" in their nitrogen requirements, for they greatly resemble Bacillus coli, although the growth is not as luxuriant. B. dysenteriae refused to grow in any of the mediums, while B. typhosus was able to develop to a slight extent in several instances only, that is, in tryptophan and several amino-acid combinations.

In view of the striking cultural resemblance of Bacterium pullorum to Bacterium sanguinarium, it is interesting to observe that the latter organism is much less exacting in its nitrogen requirements than the other. Bacterium sanguinarium developed on amino-acids, diammonium acid phosphate, and allantoin.

The proteus organisms exhibited considerable irregularity. At best their development was meager. Negative results were obtained after the introduction of limited numbers of cells. This leads one to the conclusion that the power of these organisms to utilize directly the amino-acids is exceedingly slight. Proteus zenkeri, which apparently is in no way related to the above forms (Wenner),* failed to grow in all instances.

The two strains of the cholera vibrio appear to be less exacting than the majority of the pathogenic species herein employed, for they utilized asparagin and any one of the amino-acids, although not the other sources of nitrogen. B. abortus, B. diphtheriae and B. hoffmanni exhibited no evidence of multiplication in any instance. The same may be said of all but one of the cocci studied.

By multiplying the sources of nitrogen (Table 8) it was found that most of the organisms which developed exhibited a more luxuriant growth than on the more simple mediums, especially within the first 24-48 hours. The various miscellaneous sources of nitrogen appear to be inferior to the amino-acids, although the diammonium acid phosphate and allantoin gave almost as good results.

No attempt was made to study pigment production. Incidentally, it was observed that cultures of B. pyocyaneus presented, as a rule, the characteristic deep green shade. Both types of B. fluorescens exhibited a light yellowish-green color, while the cultures of B. prodigiosus and B. ruber balticus failed to show the characteristic red pigment.

The demonstration that those organisms which are able to develop on amino-acid mediums are still enabled to do so when only a few hundred cells are introduced into each tube is especially interesting in that it indicates a power on the part of these organisms to utilize directly the amino-acids. In view of the work of previous investigators who have shown that bacteria cannot directly attack native proteins, and in some instances not even proteoses or peptones, this fact is particularly significant. It would appear that some species, at least, find immediately available nitrogen in the form of isolated amino-acids or of the other chemically-definite nitrogenous bodies employed throughout the course of this work.

It will be remembered, however, that some micro-organisms showed no indication of development on any of these substances. It is interesting to speculate as to what may be the probable nature of the nitrogenous bodies required by these species for initial development.

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* Doctorate thesis, 1918, Yale University Library.

It may be found that dipeptid or more complex polypeptids are necessary, although such evidence as exists would seem to point in the opposite direction.

Lloyd (1916) and others have suggested the necessity of "vitamins" or "accessory growth factors" for the initial cultivation of the meningococcus. The same idea has been advanced by several other writers. This opens a new field in the study of bacterial nutrition. Investigation in this field is beset with difficulties, however, since very small amounts of chemically definite nitrogenous substances may suffice to initiate bacterial development, independent of "vitamins."

A third possibility suggests itself. The nitrogen which the more "exacting" pathogens first seize on may be contained in very simple and unstable bodies which defy all methods of isolation. It seems probable that the usefulness of blood or serum, when added to ordinary culture mediums, is due to the presence of substances of this nature, substances which are readily attacked and which may pave the way, so to speak, for the attack on more complex nitrogenous bodies.

## SUMMARY

All of the amino-acids employed seem to form a rather broad group in so far as their ability to furnish nitrogen to the organisms in question is concerned. Tryptophan may be a possible exception to this.

Asparagin, the diamino-acid lysin, and the heterocyclic amino-acid histidin, apparently possess no advantage over the mono-amino acids.

A number of organisms were shown to be capable of passing through 12 successive cultures in an amino-acid medium without any diminution in the luxuriance or rapidity of development.

Those organisms which find an amino-acid medium suited to their needs are able to develop, at least to a slight extent, when very small quantities of amino-acid are supplied.

If only a few hundred cells be introduced into the amino-acid culture medium, multiplication takes place as usual and eventually the growth attained in these cultures is as luxuriant as that resulting from a much larger inoculum.

When glycerol is withdrawn from the chemically-definite mediums the bacterial development is not so luxuriant. Dextrose can be substituted for glycerol.

With the exception of the cholera vibrio, the organisms which utilize an amino-acid can as readily initiate development on diammonium acid phosphate as on the amino bodies.

Urea, taurin, creatin, hypoxanthin, and uric acid are inferior to the amino-acids as immediately available sources of nitrogen. Allantoin gives results which are quite comparable to those of the amino-acids.

Combinations of amino-acids, or of amino-acids and other nitrogen-containing compounds apparently possess little value over any one of the single amino-acids.

In a medium consisting of uric acid, together with certain mineral salts and glycerol, Bacillus aerogenes grows luxuriantly while the colon bacillus is unable to develop.

B. anthrasis, Proteus zenkeri, B. abortus, B. diphtheriae, B. hoffmanni, B. dysenteriae, and all of the cocci studied — with the exception of Sarcina lutea in a few cases — consistently failed to develop in all of the mediums employed. Bacterium pullorum developed slightly in one instance only, while B. typhosus exhibited a slight growth in a few mediums.

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